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Contemporary Problems in Biology: Cell Constituent Analysis

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P. J. Beyer, S. D. Gilman, R. A. Lee, M. R. Wood, N. Winograd, A. G. Ewing

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Department of Chemistry
Penn State University
University Park, PA 16802

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P. J. Beyer, S. D. Gilman, R. A. Lee, M. R. Wood, N. Winograd
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The adaptation of methodologies from one scientific field to another is not uncommon. Technological advances in one field can often lead to great insight toward solving the problems of another. One very successful overlap can be observed between biochemistry and analytical chemistry. In particular, methods developed in analytical chemistry have been quite beneficial to the field of neurochemistry. This interaction between the two disciplines has resulted from the miniaturization of existing analytical techniques and the development of new methods able to analyze minute environments. These miniaturized techniques can be applied to the study of cellular environments.

Several of these cellular environments are heterogeneous where each cell has its own function. Thus, the role of each cell must be determined individually. Once the specific function of each cell is understood, then its relation to other cells and to the entire organism can be determined. Only in this way can a true understanding of the chemistry of an entire organism be realized.

The considerable interest in studying single cell chemistry has resulted in the development of a number of analytical techniques. These include enzyme activity measurements, immunoassay, microgel electrophoresis, fluorescence imaging techniques, microscale ion-selective electrodes voltammetric microelectrodes, microcolumn separation techniques optical and electron microscope techniques and secondary ion mass spectrometry. Although these methods have provided valuable information, they have important limitations. Most suffer from either inadequate sensitivity, poor quantitative capabilities or an inability to monitor chemical dynamics on a time scale similar to the neurotransmission process.

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Contemporary Problems in Biology: Cell Constituent Analysis

Paula J. Beyer, S. Douglas Gilman, Roxane A. Lee, Mark R. Wood, Nick Winograd, and Andrew G. Ewing*

152 Davey Laboratory

Department of Chemistry

Penn State University

University Park, PA 16802

*Author to whom correspondence should be addressed

Introduction:

The adaptation of methodologies from one scientific field to another is not uncommon. Technological advances in one field can often lead to great insight toward solving the problems of another. One very successful overlap can be observed between biochemistry and analytical chemistry. In particular, methods developed in analytical chemistry have been quite beneficial to the field of neurochemistry. This interaction between the two disciplines has resulted from the miniaturization of existing analytical techniques and the development of new methods able to analyze minute environments. These miniaturized techniques can be applied to the study of cellular environments.[1-4]

Several of these cellular environments are heterogeneous where each cell has its own function. Thus, the role of each cell must be determined individually. Once the specific function of each cell is understood, then its relation to other cells and to the entire organism can be determined. Only in this way can a true understanding of the chemistry of an entire organism be realized.

The considerable interest in studying single cell chemistry has resulted in the development of a number of analytical techniques. These include enzyme activity measurements[5], immunoassay[5], microgel electrophoresis[6], fluorescence imaging techniques[7], microscale ion-selective electrodes[8] voltammetric microelectrodes[3], microcolumn separation techniques[3,9,10] optical and electron microscope techniques[11,12] and secondary ion mass spectrometry[13]. Although these methods have provided valuable information, they have important limitations. Most suffer from either inadequate sensitivity, poor quantitative capabilities or an inability to monitor chemical dynamics on a time scale similar to the neurotransmission process.

This chapter describes two techniques which have successfully been used to analyze single cells with either great selectivity or rapid response time. The first technique, electrochemical monitoring, is useful for detecting chemical dynamics in or at

single cells.[3] Recent advances in miniaturization of electrodes and several significant applications from biological environments are discussed. The second technique is narrow bore capillary electrophoresis (CE). CE can be used to determine a great deal about the actual chemical composition of a system because it provides rapid and highly efficient separations of ionic species in extremely small volume samples.[14] CE has been used to analyze single invertebrate[15] and mammalian[44] cells.

Electrochemical Analysis of Single Cells

Electrochemical experiments measure the current generated by a charge transfer reaction such as an oxidation or reduction when a potential is applied across two electrodes. The electrochemical experiments presented here are carried out in one of two modes, voltammetry or amperometry. In voltammetry, the potential across the electrodes is scanned from a voltage where no reaction occurs past the reaction potential while the current is monitored. Since the reaction potential is different for different analytes, qualitative information can be obtained. In amperometry, the electrode is held at a constant potential and any species which react at that potential will be detected as an oxidation or reduction current. Electrochemical analysis is limited to compounds which are easily oxidized or reduced. Fortunately, several cell components in the neurons of the brain are easily oxidized. These include dopamine (DA), norepinephrine (NE), epinephrine (E) and serotonin (5-HT).[2]

Electrodes

Recent advances in the miniaturization of electrode design has enabled analyses to be performed in small environments. There are several advantages to these small electrodes. First, voltammetry at ultrasmall electrodes is rapid and sensitive. In addition, smaller electrodes pass less current and are vitually non-destructive to the species.[2] Finally, their size allows them to be used in microenvironments including single cells.

Electrodes with ultrasmall electroactive areas have been constructed from many different materials including noble metals and carbon.[16-22] For use in biological microenvironments, electrodes must not only have small electroactive areas, but also total tip structures of micrometer or submicrometer dimensions.[3] In addition, electrodes must also be biologically compatible, since the proteins, etc. in biological media will adsorb to electrodes rendering them inactive. Carbon electrodes are the most resistant to this fouling and therefore have been most widely used.[3]

Carbon electrodes have been fabricated in the forms of disks[20], rings[21] and cylinders[22]. The carbon disk electrodes are fabricated from thin carbon fibers which are housed in glass capillaries. These electrodes generally have a total structural tip diameter of 15 to 20 μm with electroactive diameters ranging from 5 to 11 μm. This size allows them to be used outside of single cells.[20] Smaller carbon ring electrodes have been fabricated by pyrolyzing methane inside drawn silica tubes yielding total structural tip diameters as small as 1 to 5 μm. The inside of the tip is insulated with epoxy leaving a ring of carbon between the silica and the epoxy. A schematic diagram of one of these electrodes is shown in Figure 1 (Top). These electrodes are useful for both intracellular and extracellular voltammetry.[21]

Cylindrical carbon fiber electrodes have also been fabricated from carbon fibers with diameters of 5 to 11 μm. In this case, however, individual fibers protrude from the glass housing and can be etched to tips as small as 200 nm. The sides of the fiber must still be insulated in order to constrain the electroactive area to the tip of the electrode. This insulation tends to increase the overall structural diameter. Insulating films of 100 nm thickness have been obtained by electropolymerization of phenol and 2-allylphenol.[23] Electrodes with total structural diameters as small as 400 nm have been constructed. A schematic of this type of electrode is shown in Figure 1 (Bottom).

Monitoring Exocytosis

The release of neurotransmitters from single cells has also been examined using electrochemical detection. In this type of analysis, a microelectrode is placed inside of, on top of, or directly next to a cell. The cell is stimulated and the release of the easily oxidizable neurochemicals, the catecholamines, is monitored. The rapid response times of these electrodes are well suited for the detection of these discrete neurochemical events.[24]

Mammalian neurons are difficult to work with in the laboratory as they stop proliferation once they have matured. Green and Tischler established a nerve growth factor responding clonal line of rat Pheochromocytoma (PC12) cells in 1976. This cell line shares many physiological properties with primary cultures of sympathetic ganglion neurons[25], and has been studied as a model for the developing sympathetic nerve.[26] PC12 cells can synthesize, store and release catecholamines in a manner similar to sympathetic ganglion neurons.[25, 26] They are more similar to sympathetic ganglion neurons than to adrenal chromaffin cells, because they contain more dopamine than norepinephrine with no detectable level of epinephrine.[27] Most importantly, PC12 cell vesicles are valid analogs of brain synaptic vesicles for four criteria: size, density, protein composition, and endocytotic origin.[26, 27]

Carbon fiber electrodes have been used to monitor single exocytotic events from individual PC12 cells. The electrode is placed against the top of a cell in culture and the cell is stimulated in a manner to that described above for adrenal cells with KCl or nicotine. Figure 2 shows the amperometric response following three successive stimulations with both 1 mM nicotine and 105 mM KCl. Stimulated release is not observed from these cells if calcium is omitted from the medium. In addition, preliminary data suggests that exposure to nicotine alone results in minimal exocytosis and that is only after a substantial delay. Exposure to elevated potassium chloride causes most of the exocytosis observed. Individual current spikes have an average half width of 9.3 ± 0.1 ms (n=1912 from 13 cells) and the average catecholamine level calculated with Faraday's law

is 190 zmol (114,000 molecules) per vesicle. Monitoring zeptomole levels of catecholamine from single exocytotic events provides the means to monitor physiological and pharmacological alterations of quantal size. Each vesicle has attoliter volume so these measurements represent an extreme example of measurements concerning small environments.

Microcolumn Separations of Single Cells

Open Tubular Liquid Chromatography and Microbore LC

Open tubular liquid chromatography (OTLC) and microbore LC separations are carried out in 1 to 50 μm inner diameter (i.d.) capillaries 1 to 3 m in length with a stationary phase bound to the inner wall (OTLC) or to micrometer sized particles (microbore LC).[3] Separation of analytes is then based on the differential retention of analytes by the stationary phase. This type of separation exhibits high resolution with low sample volumes, and is also useful for both quantitative and qualitative analysis. Electrochemical and fluorescence detection methods are the most sensitive for OTLC.[9,28-31]

Capillary Electrophoresis

Capillary electrophoresis is a relatively new separation technique which is well suited for the analysis of single cells. In CE, a solution filled capillary is suspended across two buffer reservoirs with a potential applied across them. A sample CE apparatus is shown in Figure 3. When a potential is applied, ionic solutes begin to migrate in the capillary; positive ions will migrate toward the negative end of the capillary, and visa versa. This movement is known as electromigration. The rate at which the species migrate is proportional to their mass to charge ratio. If this were the only force acting in CE, anions and cations would elute at opposite ends of the capillary; however, the presence of another force, electroosmotic flow (EOF) results in all ions eluting at the anodic end of the capillary. Electroosmotic flow is the bulk movement of liquid through the capillary when a potential field is applied. This is due to the build up of cations next to

the negatively charged wall.[32] As a potential is applied, the cations migrate toward the anode and drag bulk solvent along with them. This results in a flow profile which has a flat velocity distribution across the center and causes highly efficient separations.[33] One of the main advantages of EOF is that it is strong enough to cause elution of cations, neutrals, and anions at the same end of the capillary.

The capillaries used for CE range from 10 to 100 cm in length and 2 to 100 μm inner diameter (i.d.). Such small capillary bores have very high surface area/volume ratios which allow for rapid heat dissipation through the capillary wall; therefore, high separation potentials (30 kV / m) can be used to speed up the analysis.[33]

Injection Techniques

The total volume of a 5 μm i.d., 100 cm long capillary is only on the order of 20 nL, so introduction of very narrow sample plugs is necessary. Two of the injection techniques used with CE which are effective for introduction of such low volumes are hydrodynamic injection and electrokinetic injection. Hydrodynamic injection is implemented through the application of a pressure differential across the length of the capillary. The pressure differential can be generated by raising the sample end of the capillary relative to the detection end, by pressurizing the sample container or by creating a vacuum at the detection end of the capillary. The other method, electrokinetic injection utilizes the same electromigration process that is used to separate the ions. In this process, the cathodic end of the capillary is placed directly in the sample as a voltage is applied for a few seconds. Electrokinetic injection is particularly useful for biological samples due to the minimization of sample handling and dilution. The simplicity of this technique has caused it to be used frequently; however, it does have some drawbacks. One problem is that a charge bias is induced. During the injection time, cations will move farther ahead than anions. This causes a difference in the apparent injection volume of the two species, however the actual injection volume can be determined if the electrophoretic mobility is known.

Detection Techniques

Many different detection methods have been used with CE including absorption, fluorescence, mass spectrometry, and electrochemistry.[14] Of these, the two most sensitive methods are electrochemical and fluorescence detection. Electrochemical detection (EC) has been demonstrated in both the amperometric and voltammetric modes.[34] Amperometric methods are more sensitive; however, voltammetric detection can result in more qualitative information. In CE-EC experiments, carbon microelectrodes are placed either directly outside of the capillary or in the anodic end of the separation capillary.[34, 35] Capillaries as small as 2 μm i.d. have been used with electrochemical detection after a conical section has been etched in the detection end of the capillary with hydrofluoric acid.[36] Detection limits of 19 amol of catechol have been obtained under these conditions. The best detection limits for catechol using a 5 μm i.d. capillary for off-column and end column detection are 0.6 amol and 56 amol, respectively.[15, 36]

Laser induced fluorescence detection (LIF) is also compatible with CE of biological samples.[37-41] In CE-LIF, excitation is induced by focusing a laser to a small spot on a window in the separation capillary. The lasers used most frequently are helium cadmium and argon ion lasers at wavelengths of 325 nm and 488 nm, respectively. Fluorescence emission is collected perpendicular to the incident light to minimize the influence of stray light. Since most species in a cell are not inherently fluorescent, derivatization is necessary. Pre-and post-column derivatization schemes have been demonstrated using the fluorophores naphthalenedicarboxyaldehyde (NDA), o-phthaldialdehyde (OPA), fluorescein isothiocyanate (FITC), and 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA).[41]

Pre-column derivatization is the simplest method; however, multiple derivatization sites of a single analyte may lead to band broadening or even several peaks for the same analyte. In addition, migration times for the analyte and the derivatized product are different and may cause difficulty in identification. Post-column derivatization avoids

these problems by allowing the separation of analytes to occur before derivatization. In this case, however, band broadening and derivatization reaction time become issues.[40-42] Detection limits of 10^{-15} - 10^{-21} moles have been reported depending upon the fluorescent tag used.

Indirect fluorescence is a possibility for detection of species which cannot be derivatized. In this case, the sample buffer is fluorescent and the analyte is recognized by a decrease in signal as the fluorophores are displaced. Indirect detection is less sensitive than direct LIF, but it does offer the opportunity to detect inorganic species such as sodium or potassium.[10]

Whole Cell Analysis

OTLC and Microbore Column Analysis

Jorgenson and co-workers have demonstrated single cell analysis using OTLC with voltammetric detection.[28-30] They separated the components of homogenized giant neurons of the land snail *Helix aspersa* and identified tyrosine, tryptophan, dopamine, and serotonin in these cells.[29] These experiments have been used to substantiate coexistence of more than one neurotransmitter in a single neuron. OTLC with amperometric detection and NDA derivatization has also been used for single cell analysis.[30] This analysis proved useful for amino acids; however, the neurotransmitters DA and 5-HT were lost during sample preparation.

Single mammalian cells from adrenomedullary tissue have also been homogenized and separated with liquid chromatography combined with electrochemical detection. However, in this separation, packed microcolumns were used rather than open columns since the open columns do not provide enough retention for polar compounds such as NE.[31] The existence of two types of catecholamine secreting cells, NE-rich and E-rich, in mammalian adrenomedullary cells is generally accepted.[31] The contents of 22 cells from 5 cell preparations were quantified with the micro-LC-EC technique. It is interesting to note that all of these E-rich cells contained NE (detection limit 46 amol) although several

of the NE-rich cells did not contain E (detection limit 75 amol). Several cells contained significant amounts of both neurotransmitters which could indicate a third type of cell in this tissue or that cells can convert back and forth between the two cell types.[31]

CE of Invertebrate Cells

Jorgenson and co-workers used CE with LIF detection and derivatization with NDA to analyze homogenized neurons from the land snail, *Helix aspersa*. In these experiments only about 20% of the cell was analyzed at a time. This allowed an assessment of the reproducibility of the results since multiple runs of the same cell could be performed.[9] However, this requires a significant amount of sample preparation.

Whole cells can also be injected directly on to the capillary by electrokinetic injection then broken down, or lysed, inside the capillary. The lysing procedure involves injection of a non-physiological buffer immediately after cell injection followed by a 60 s or longer lysing time. In this way, the entire contents of a cell can be analyzed at once.

Kristensen et al.[43] used CE-EC with electrokinetic injection to analyze the giant dopamine cell from the pond snail, *Planorbis corneus*. Separations of cell components resulted in peaks for uric acid (UA) and dehydroxyphenylacetic acid (DOPAC) and what appears to be two peaks for dopamine. The second dopamine peak is dependent upon the lyse time is diminished by the vesicle-depleting drug, reserpine. These results indicate a 2-compartment model for dopamine in these cells. The first peak represents the functional, or releasable dopamine seen on stimulation of the cell. The second peak represents the dopamine found in storage vesicles which are non-functional, and are not released until the cell is completely lysed.[43]

CE of Red Blood Cells

One of the smallest cells analyzed to date by CE is the single human erythrocyte, or red blood cell.[10, 37, 44] This cell is about 8 to 9 μm in diameter and has a total cell volume of about 90 fL. Yeung and co-workers isolated these cells from whole blood and examined them using CE with both direct[37] and indirect fluorescence detection.[10] The

direct fluorescence scheme was used to determine the level of the peptide, glutathione (GSH). GSH is suspected to play a role in the cellular response to various types of drugs and radiation. As GSH is not inherently fluorescent, the cells were derivatized with monobromobimane (mBBr) before sampling. Derivatization was initiated by allowing the mBBr to diffuse through the semipermeable cell membrane.[37] The derivatized product is unable either due to size or change in hydrophobicity to migrate back through the membrane. A derivatized cell was then hydrodynamically injected into a 10 μm i.d. capillary, lysed and separated. Levels of 68±48 amol of GSH have been reported. The large error shows the great variability between cells and reiterates the importance of looking at such phenomena on the single cell level.

Fluorescence detection without derivatization has been demonstrated for the natively fluorescent proteins, hemoglobin A_0 (HemA) and carbonic anhydrase (CAH) in red blood cells. Values of 5 amol (CAH) and 0.47 fmol (HemA) have been determined with this method and these values are very similar to the standard literature values.[37]

Indirect fluorescence detection has been used to determine the amount of sodium and potassium in single erythrocyte cells (Figure 4), since these inorganic ions have no good derivatization schemes. A fluorescent buffer containing 6 aminoquinoline and cetyltrimethylammonium bromide is used and the Na^+ and K^+ ions are detected by the charge-displacement effects of the buffer. This method of detection is not as sensitive as direct detection and therefore cannot currently be used for analysis of less concentrated species.[10]

CE of Mammalian Lymphocytes

Using CE-EC, the presence of catecholamines and their metabolites in single lymphocytes and extracts of T-cell clones has been detected.[45] A CE separation of a single lymphocyte contains a peak with a migration time of 10.75 min that has tentatively been identified as dopamine. Similar electropherograms are obtained for cloned $CD4^+$ T cells. CSF lymphocytes have been observed to contain 2.3 ± 1.7 attomoles of

catecholamine and cloned $CD4^+$ T cells have been observed to contain 31 ± 29 attomoles. In one lymphocyte, 310 zeptomoles of catecholamine were detected demonstrating the extreme sensitivity of this methodology.

To study the uptake and metabolism of catecholamines in clones of $CD4^+$ T lymphocytes, the effects of incubating T-cell clones with either dopamine or the dopamine systhesis inhibitor, α-methyl-p-tyrosine (α-MPT) were examined. Following incubation, the cultured cells were extracted and a sample was examined by capillary electrophoresis. For the cells incubated with dopamine, a large increase in the level of catecholamine per cell is observed. Conversely, incubation with α-MPT decreases the level of catecholamine in cloned $CD4^+$ T lymphocytes. The data for dihydroxyphenylacetic acid follow this general trend. Experimentally, these data strongly suggest that the catecholamine peak is indeed dopamine and that this catecholamine is both sythesized and accumulated by lymphocytes.[45] This is the first evidence of the presence of catecholamines in lymphocytes.

Cytoplasmic Analysis

Neurotransmitters can be found in two different places in neurons--free (cytoplasmic) and bound (vesicularized). Previous analyses have focused on the bound neurotransmitters, so investigations of the neurotransmitter content in the cytoplasm should lead to a better understanding of the role of the cell in neurotransmitter uptake, storage and metabolism. Detection of cytoplasmic levels of the neurotransmitter, dopamine, became possible with implementation of direct electrokinetic injection with 2 and 5 μm i.d. etched capillaries.[15, 46, 47] Capillaries were etched in hydrofluoric acid to outer diameters of 6 to 10 μm which made them small enough to place through the cell membrane directly into a neuron. A small amount (50 pL) of the cytoplasm was sampled and separated. The systems examined were the giant dopamine and serotonin nerve cells of the pond snail, *Planorbis corneus*. The DA cell is about 200 μm in diameter with a

total cell volume of about 5 nL. The injection volume of 50 pL is only about 1% of the total cell volume. Cytoplasmic levels of 5-HT were found to be 3.1±0.57 μM and that of DA 2.2±0.52 μM.[15, 46, 47]

Future Directions

Picoliter Reaction Vials

Future directions for single cell analysis include further improvements in sample handling and derivatization schemes. One possibility is the use of picoliter vials as reaction vessels and sampling wells. Square pyrimidal vials ranging in size from 8.5 pL to 95 pL have been fabricated with the assistance of the National Nanofabrication Facility at Cornell University. These vials are inverse square pyramids etched in silicon using standard photolithographic techniques. A scanning electron micrograph of a 50 pL vial is shown in Figure 5. Arrays of 2500 wells of 4 different sizes have been etched on a three inch silicon wafer. These are spaced 1 mm apart to allow for eventual automation of injection procedures. A thin layer (1000 Å) of gold with a 70 Å chrome adhesion layer is deposited over the entire structure to serve as an electrophoresis anode for injection. Larger vials, 118 nL, have been used previously for similar purposes.[48]

One purpose of these vials is to limit the dilution of the sample either upon derivatization or stimulated release of the cell. By confining such release to a known volume, more accurate quantitation of the release should be possible. Using CE and the smaller vials, the entire volume can be injected and separated.

Initial characterization of the vials to determine the effects of evaporation, capillary action, contamination and adsorption has been accomplished. Evaporation from these vials can be a significant problem. Complete evaporation from the 95 pL vials occurs in minutes in the open laboratory. Since the smaller vials have the same surface area / volume ratio, the evaporation rate should be constant and the smaller vials will be evaporated to dryness even faster. We have found that adding 15% glycerol by volume to the solutions successfully slows evaporation to a usable rate and all successful experiments

carried out to date have used this protocol. The successful implementation of picoliter microvials affords the possibility to localize extremely small quantities of material with great spatial resolution.

Picoliter microvials have been used in conjunction with time-of-flight secondary ion mass spectrometry imaging to localize and quantitate test substances with micrometer coordinates and submicrometer resolution. In these experiments, a 10^{-4} M solution of crystal violet was injected into a 50 pL vial and the solvent allowed to evaporate completely. A TOF SIMS image of the crystal violet molecular ion (m/e = 372) is shown in Figure 6. The square shape of the vial can be clearly observed with very little crystal violet located outside the vial. The total imaged area is 100 x 84 um with 0.33 x 0.33 um pixel resolution. This level of resolution is obtained by use of a gallium liquid metal ion gun with a tightly focussed ion beam. TOF SIMS images have also been obtained inside and outside microvials monitoring total ion count, sodium ion (contamination) and the gold coating that overlays the vials and the surrounding silicon wafer (data not shown).

Using gold as a substrate, requires that its contamination be considered. Gold is contaminated fairly readily in the atmosphere. Adsorption of organic species from the air occurs on the order of a few minutes.[49] This causes the gold surface to become more hydrophobic and suggests the possibility that the sides of the vials might repel the solution. Observations by optical microscopy indicate that, particularly when glycerol is added, this is not the case. Hence, the microvials have been used for liquid phase sampling at the picoliter level for subsequent capillary electrophoresis separation.

A preliminary separation of five easily oxidized catecholamine standards from a 95 pL vial is shown in Figure 7. A solution consisting of 10^{-5} M dopamine, epinephrine and catechol with 10^{-4} M uric acid and DOPAC (15% glycerol by volume) was injected into a vial using pressure injection and then electrokinetically injected into the etched end of a 5 μm i.d. separation capillary. Electrochemical detection was used to identify the species by comparisons with the electromigration times of the standards from a larger vial under

identical conditions. The peaks from the picoliter vials in all cases were smaller than the pure standards. This might result from analyte adsorption onto the gold surface of the vial. The sample injection volumes were estimated to be about 75% of the total vial volume. Due to the pyramidal shape of the vials, the injector is unable to reach the bottom as the opening narrows with depth. Therefore the solution in the point at the bottom of the pyramid is not necessarily sampled.

Derivatization in a picoliter vial has also been attempted. In these experiments 45 pL of an amino acid solution was injected into a 95 pL vial immediately followed by a 45 pL injection of NDA. The amount of NDA was twice the amount needed for complete reaction, and a reaction time of approximately 10 min was used before separation. The results of this separation are shown in Figure 8.

Experiments with picoliter reaction vials are still in the characterization stage. the eventual goal is to use these vials to facilitate single cell measurements. Electrochemical monitoring of the neurotransmitter release from single vesicles upon stimulation have been described above. We plan to carry out these experiments on cells immobilized in picoliter sampling wells. This should permit the total quantitation of easily oxidized substances released via exocytosis from a single nerve cell in culture. Fast scan voltammetry (300 V/s) in ferrocene containing solutions has been used to show that these voltammetric analyses will be plausible in the future.

Post-column Reactors for CE-LIF

Another future development is the optimization of post-column reactors for CE-LIF. As previously mentioned, post-column derivatization allows the analytes to migrate at their own electrophoretic mobilities without interference by the derivatization agent. Post-column techniques have generally used OPA or fluorescamine to derivatize primary amines in amino acids, proteins and biological polyamines.[50, 51] Designs for post-column reactors using pressure driven mixing or mixing due to electroosmotic flow have been reported.[50, 51] Most of these designs have used capillaries in the range of 25 to 50 μm i.d.

A smaller capillary is generally used for single cell analysis, therefore a reactor using a 10 μm i.d. capillary was constructed.[52]

This reactor used the same 10 μm i.d. capillary for the separation and detection segments of the capillary with a narrow gap in between. The gap distance between the two capillary segments ranged from 4 to 160 μm. The best results were obtained with the smaller gap distance (<10 μm). A reagent reservoir was formed over the gap to allow more control in the introduction of the derivatizing reagent. A schematic of this design is shown in Figure 9 and a separation of separated and derivatized amino acids and proteins in Figure 10. Derivatizing reagent introduction is mainly controlled by diffusion in the gap. The mass sensitivity for amino acids and proteins is better in this system than in other post-column derivatization systems.[52]

In summary, many advances have been made in developing nanoscale tools to understand what occurs in biological microenvironments. Advances still need to be made in detection sensitivity, selectivity and response time. In addition, electrodes with considerably smaller tip diameters (approximately 50 nm) will be required. These constitute the major challenges in nanofabrication for analytical chemistry in biological microenvironments.

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Figure Legends

Figure 1. Schematics of two different carbon voltammetric electrodes. (Top) Carbon ring electrode. (Bottom) Etched and insulated carbon fiber electrode.

Figure 2. Amperograms of single vesicular exocytosis from PC12 cells. (Top) Amperogram of vesicular exocytosis induced by 1 mM nicotine in 105 mM K^+ balanced salt solution. (Bottom) Enlargement of a one second period of the amperogram from the first stimulation. Data displayed correspond to the time period near the middle of the first base line disturbance in (Top).

Figure 3. Schematic of CE system. From Ewing, A. G.; Wallingford, R. A.; Olefirowicz, T. M. (1989) *Anal. Chem.* 61:294A. With permission.

Figure 4. Electropherogram using indirect fluorescence detection. (A) Injection of 45 μ M standards. Peak 1 is Li (11.7 fmol injected), peak 2 is Na (10.7 fmol), and peak 3 is K (6.5 fmol). (B) Injection of 1 human erythrocyte. (C) Blank injection of extracellular matrix. From Hogan, B. L., Yeung, E. S., (1992) *Anal. Chem.* 64:3045. With permission.

Figure 5. Reproduction of a scanning electron micrograph of a 50 pL microvial. Scale bar is 10 μm.

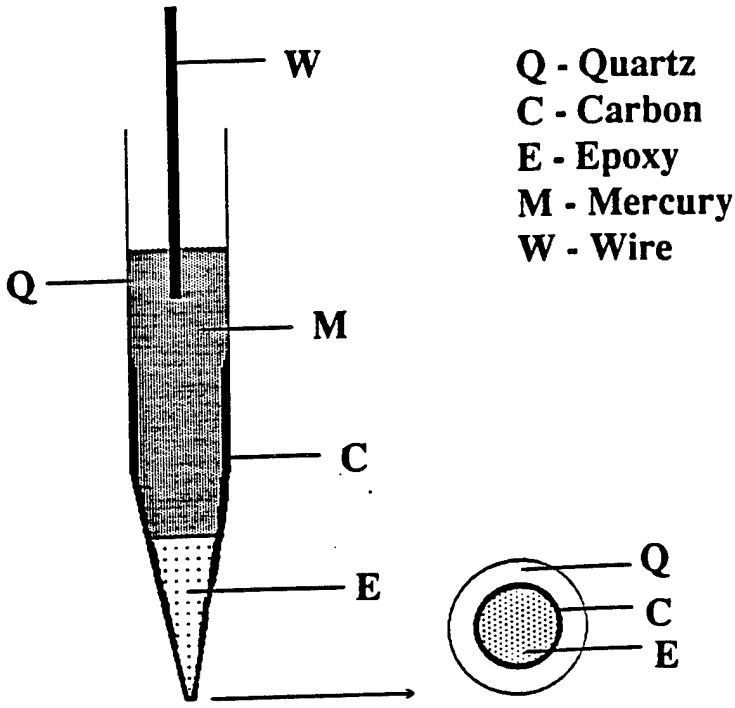
Figure 6. SIMS image of 10^{-4} M crystal violet in a 50 pL microvial. Imaged area is 100 x 84 μm taken with pixel resolution of 0.33 μm x 0.33 μm. The ion dose to the surface was 1.7×10^{12} ions / cm.

Figure 7. Trial separation of standards sampled from a 95 pL microvial with electrochemical detection. Injection: 10 s, 10 kV; 84.1 cm, 5 μm i.d. capillary; separation voltage: 30 kV.

Figure 8. Trial separation of standards sampled from a 95 pL microvial with fluorescence detection. Injection: 8 s, 2 kV (60 pL); 100-cm, 10-μm i.d. capillary etched to 25 μm tip; separation voltage: 30 kV; derivatization: 15 min reaction of 45 pL NDA (1 mM); KCN (1 mM) with 45 pL amine sample.

Figure 9. Drawing of a CE system with a post-column reactor and a laser fluorescence detector.

Figure 10. Separation of three proteins and three amino acids using post-column derivatization and LIF detection. Peak identities and injected amounts are: A, DL-arginine hydrochloride ($5x10^{-5}$ M, 9.8 fmol); B, horse heart myoglobin (0.5 mg/mL, 4.1 fmol); C, iron-free human transferrin (0.25 mg/mL, 450 amol); D, bovine serum albumin (0.5 mg/mL, 880 amol); E, (L+)-glutamic acid (5x10-5 M, 3.5 fmol); F, DL-aspartic acid, ($5x10^{-5}$ M, 3.2 fmol). Conditions: capillary, 10 μm i.d., 100 cm length, 80 cm to gap and 82.5 cm to detection window; 4 μm gap; injection, 5 s, 10 kV (182) pL; separation potential, 30 kV (300 V/cm); buffer, 100 mM borate, pH 9.5; current 1.0 μA.



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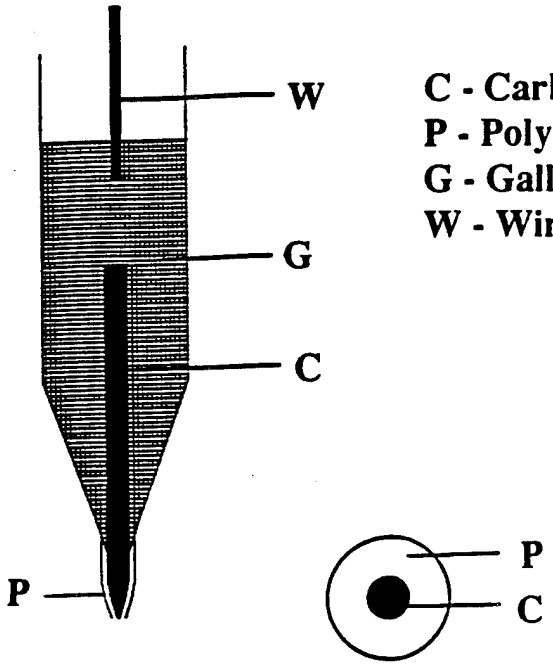
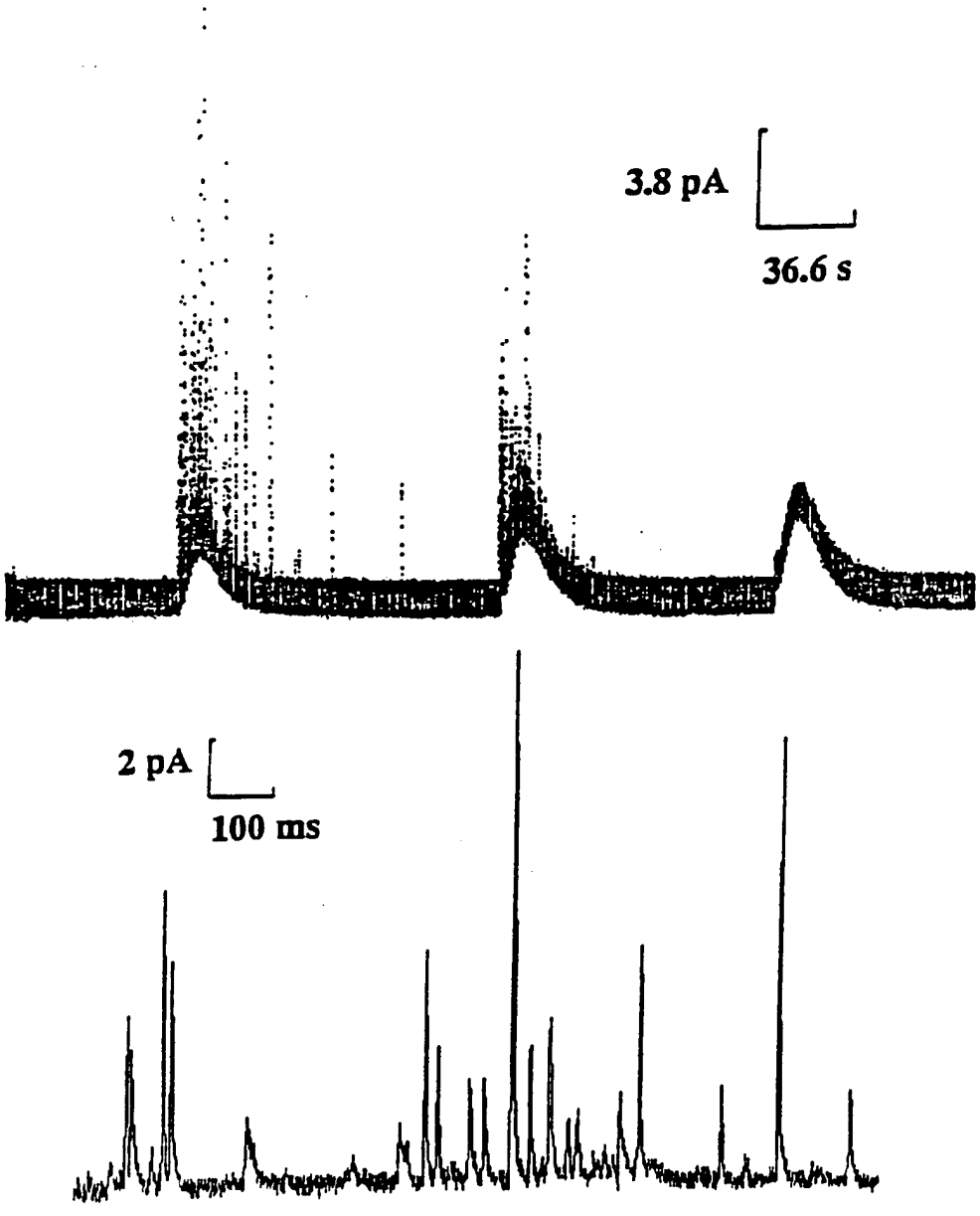
C - Carbon
E - Epoxy
M - Mercury
W - Wire



C - Carbon
P - Polymer
G - Gallium
W - Wire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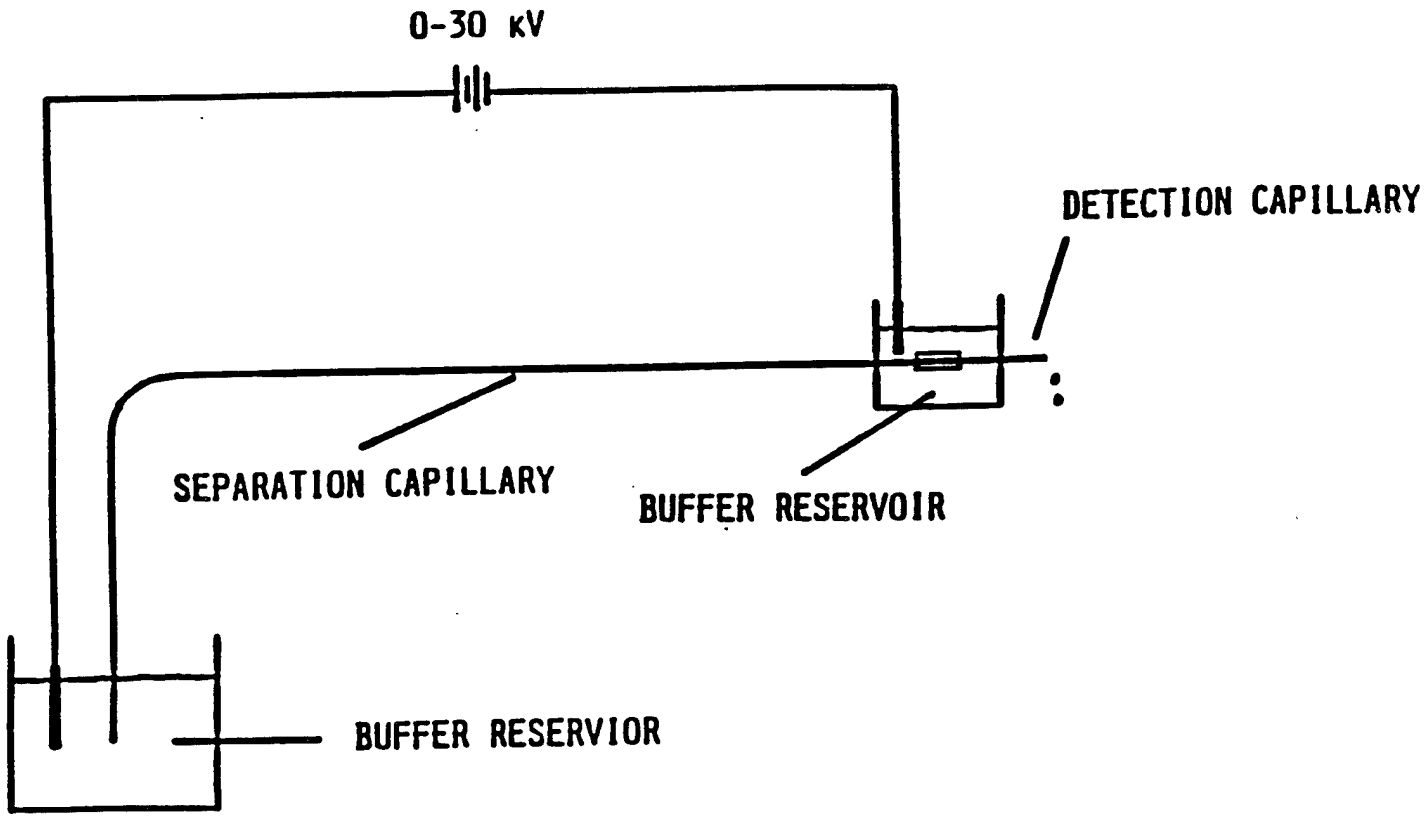
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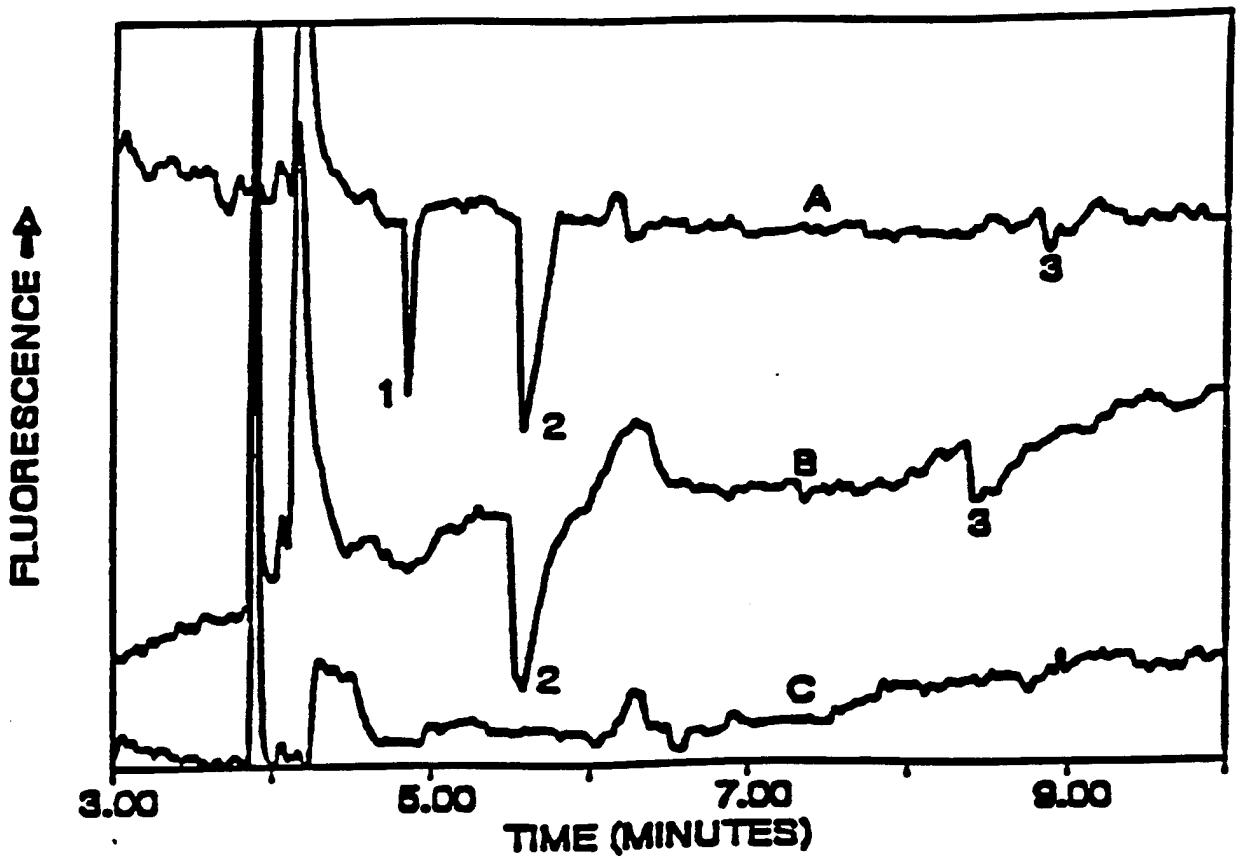
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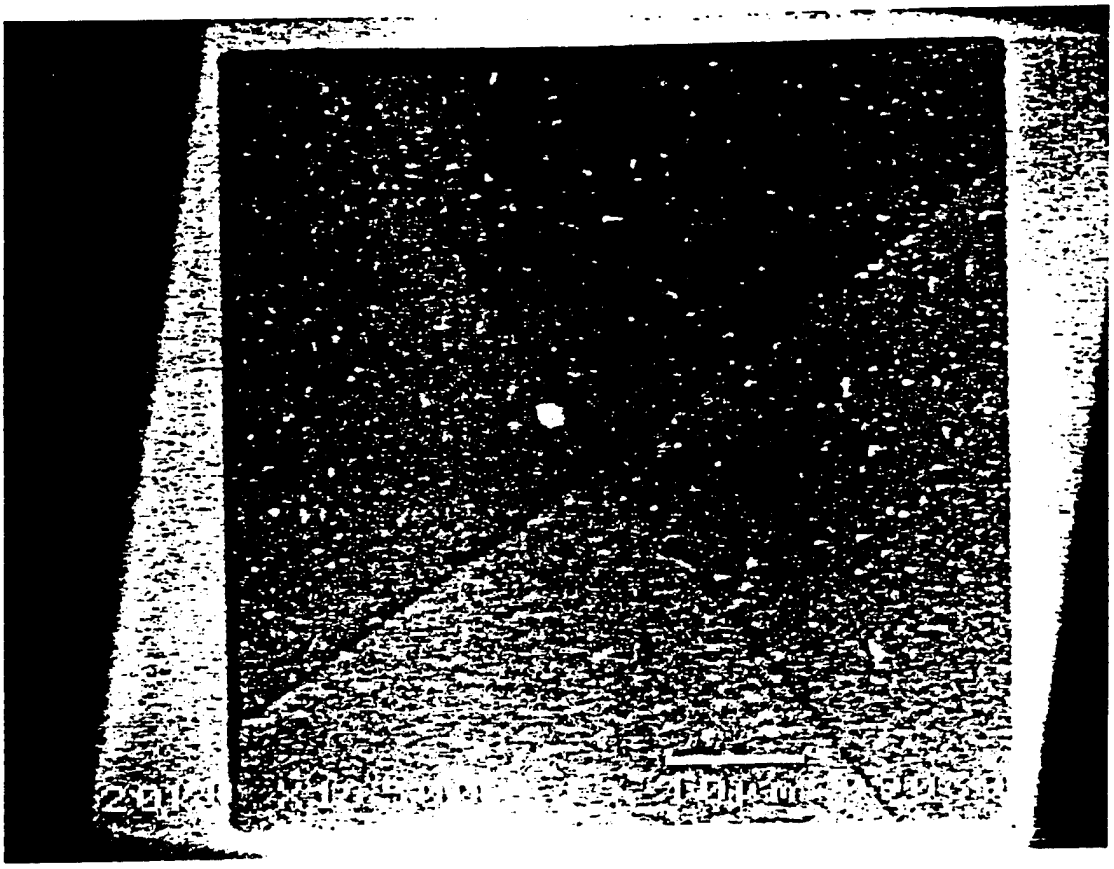

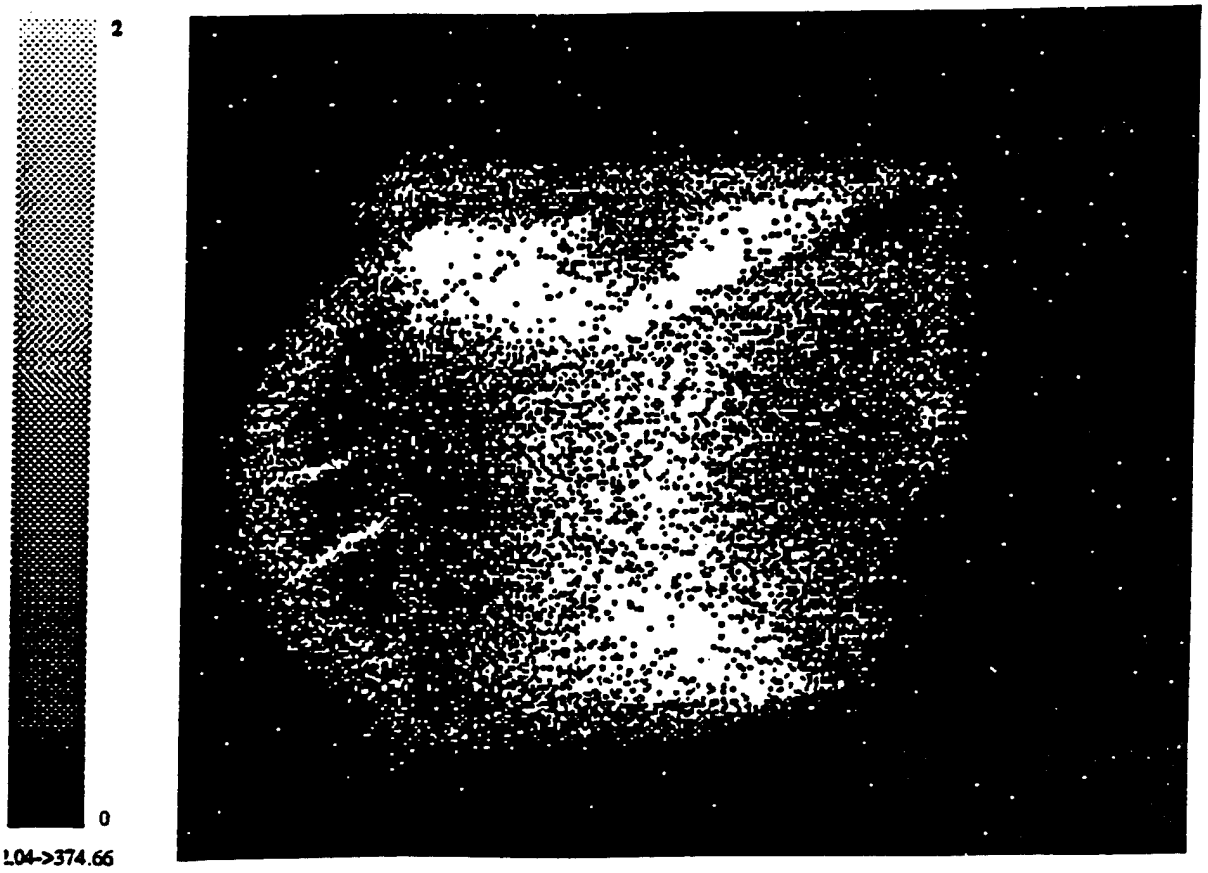
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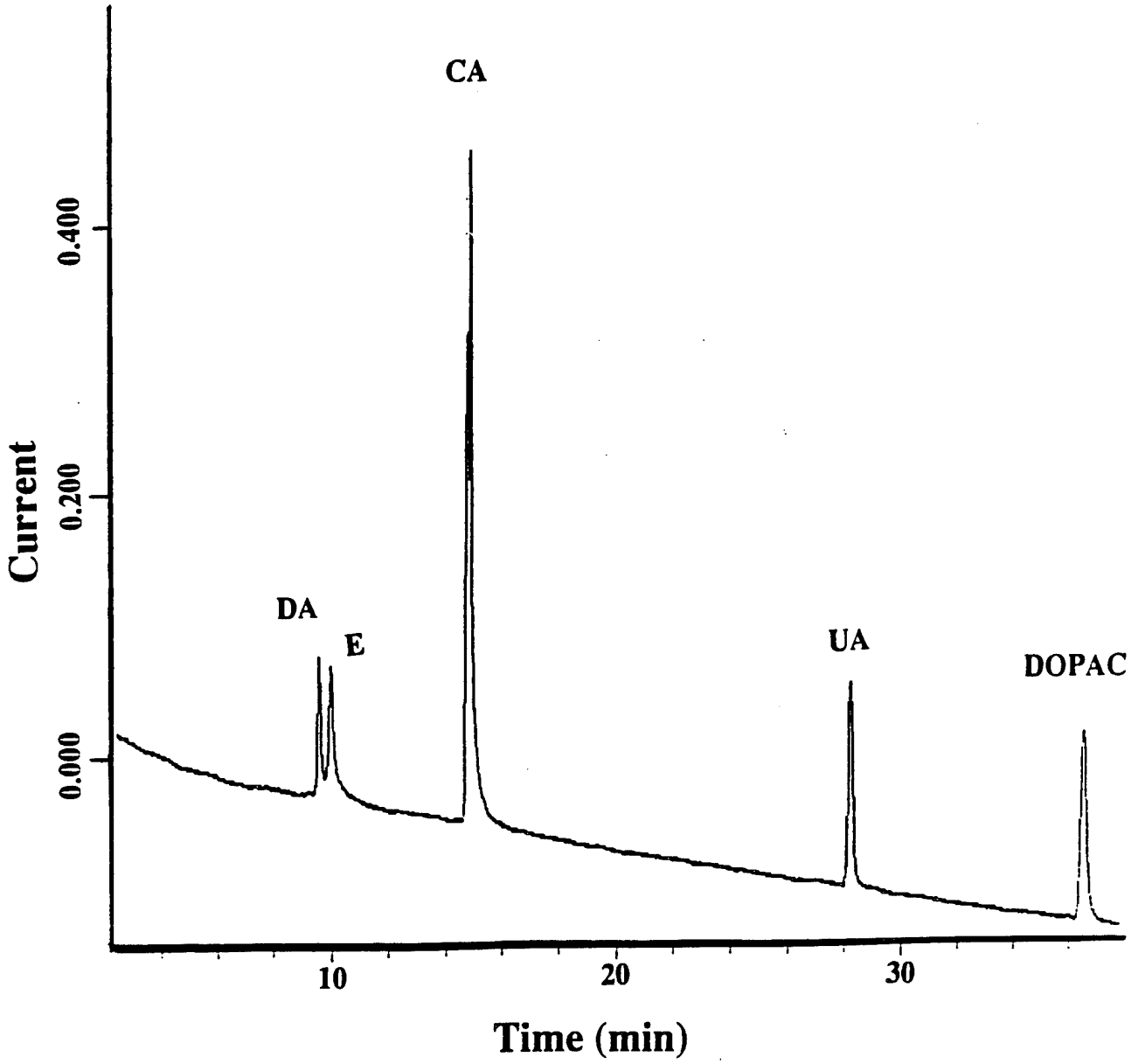
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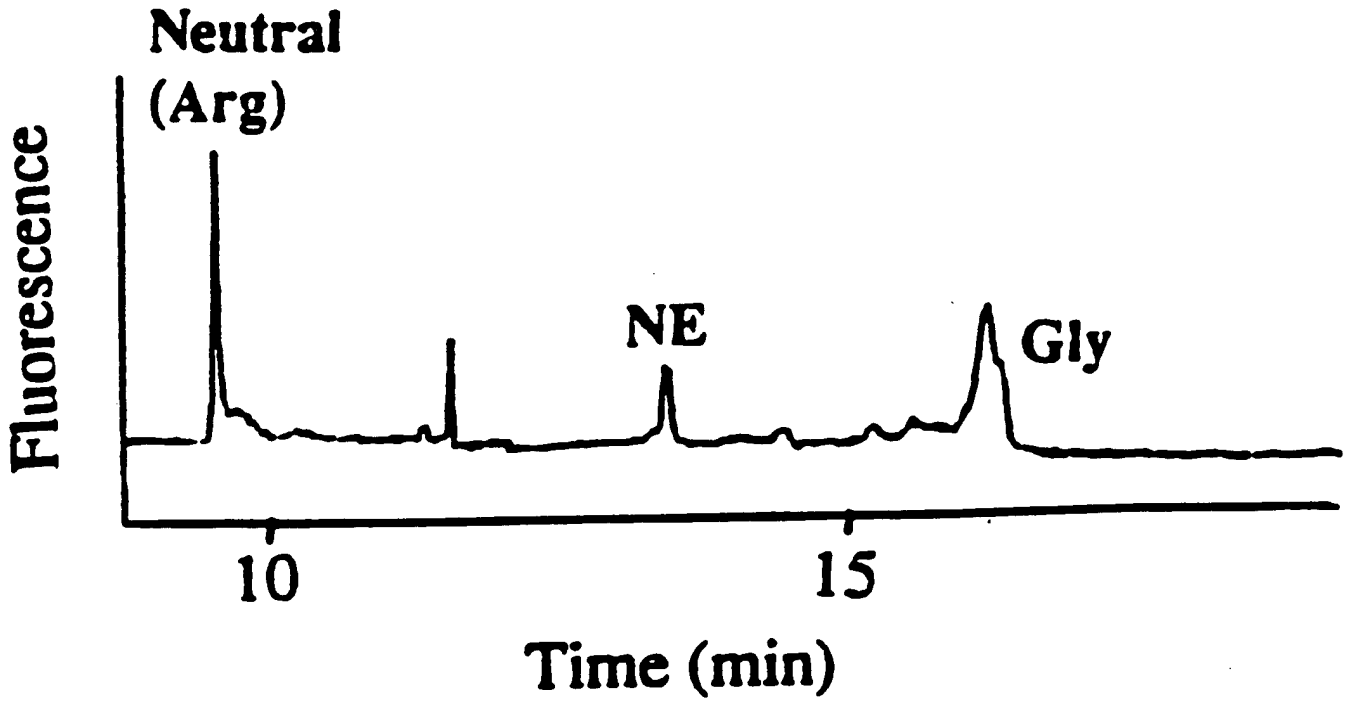
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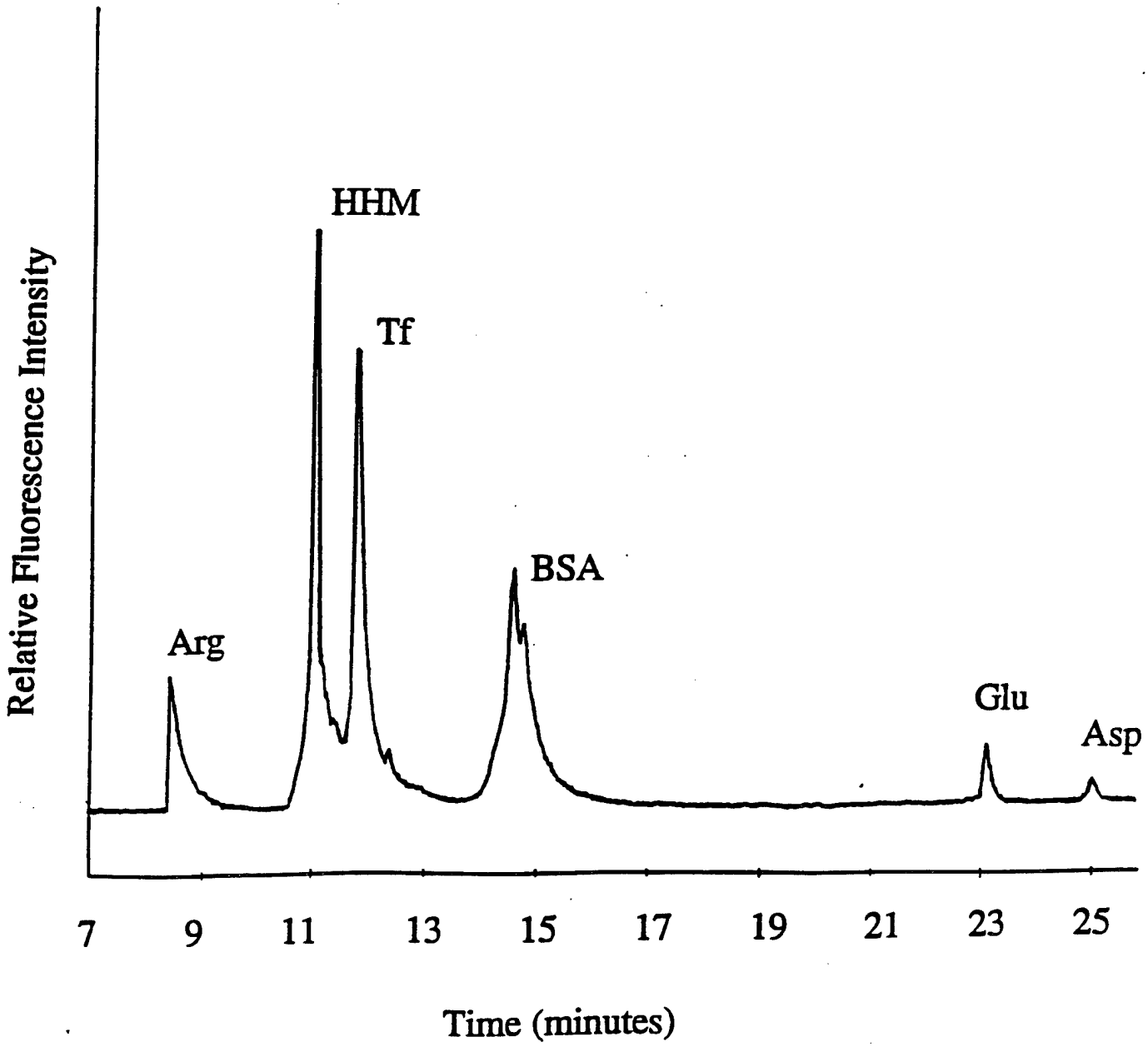
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